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(71) Applicant: CELL GENESYS, INC. [US/US]; 342 Lakeside Drive, Foster City, CA 84404 (US).

(72) Inventors: COHEN, Lawrence, K.; 10065 Broadway Terrace, Oakland, CA 94611 (US). SPRATT, S., Kaye; 104 Edmondale Court, Vacarille, CA 95688 (US). COUTO, Linda; 7814 Oak Creek Drive, Pleasonton, CA 94588 (US).

(74) Agents: NAKAMURA, Dean, H. et al.; Sughrue, Mion, Zinn, MacPeak & Seas, PLLC, Suite 800, 2100 Pennsylvania Avenue, N.W., Washington, DC 20037-3202 (US).

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(54) Title: ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED EXPRESSION OF FACTOR VIII ACTIVITY

(57) **Abstract**

The instant invention provides methods and materials for expressing polypeptides with factor VIII activity comprising administering at least two rAAV vectors encoding different domains of human factor VIII and at least the heavy and light chains.

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ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED EXPRESSION OF FACTOR VIII ACTIVITY

BACKGROUND OF THE INVENTION

I. ADENO-ASSOCIATED VIRUS

Adeno-associated virus (AAV) is a defective parvovirus whose genome is encapsidated as a single-stranded DNA molecule. Strands of plus and minus polarity are packaged with equal efficiency, but in separate virus particles. Efficient replication of AAV generally requires coinfection with a helper virus of the herpesvirus or adenovirus family, although under special circumstances, AAV can replicate in the absence of helper virus.

In the absence of helper virus, AAV establishes a latent infection in which the viral genome exists as an integrated provirus in the host cell. Integration of the virus occurs on human chromosome 19. If a latently infected cell line later is superinfected with a suitable helper virus, generally the AAV provirus is excised and the AAV virus enters the "productive" phase.

AAV isolates have been obtained from human and simians. The host range for lytic growth of AAV is broad. Cell lines from virtually every mammalian species tested, including a variety of human, simian, canine, bovine and rodent cell lines, can be infected productively with AAV, provided an appropriate helper virus is used (e.g., canine adenovirus in canine cells). However, no disease has been associated with AAV in either human or other animal populations.

AAV has been isolated as a nonpathogenic coinfecting agent from fecal, ocular and respiratory specimens during acute adenovirus infection, but not during other illnesses. Latent AAV infections have been identified in both human and nonhuman cells. Overall, virus integration appears to have no apparent effect on cell growth or morphology, see Samulski, Curr. Op. Gen. Devel., 3:74-80 (1993).

There are a number of AAV's, including AAV-1, AAV-2, AAV-3, AAV-4 and AAV-5. The genome of AAV-2 is 4,679 bases

in length (Genbank No. AF043303) and contains inverted terminal repeat sequences of 145 bases each. The repeats are believed to act as origins for DNA replication. The AAV-3 genome is 4726 bases in length and has 82% overall sequence homology with AAV-2, see Muramatsu, Virology, 221:208-217 (1996). Like AAV-2, both ends of the AAV-3 genome consist of inverted repeats but the palindromes are 146 bp in size. Certain portions of the AAV-2 and AAV-3 genomes are highly conserved, for example, there are two sites in the hairpin where there is only a single base pair substitution between AAV-2 and AAV-3.

The AAV genome has two major open reading frames. The left frame encodes at least four non-structural proteins (the Rep group). There are two promoters, P5 and P19, which control expression of those proteins. As a result of differential splicing, the P5 promoter directs production of proteins Rep 78 and Rep 68, and the P19 promoter, of proteins Rep 52 and Rep 40. The Rep proteins are believed to be involved in viral DNA replication, trans-activation of transcription from the viral promoters, repression of heterologous enhancers and promoters as well as with site-specific integration.

The right ORF, controlled by the P40 promoter, encodes the capsid proteins, Vp1 (91 kDa), Vp2 (72 kDa) and Vp3 (60 kDa). Vp3 comprises 80% of the virion structure while Vp1 and Vp2 are minor components. There is a polyadenylation site at map unit 95. For the complete sequence of the AAV-2 genome, see Strivastava et al., J. Virol., 45:555-64 (1983).

McLaughlin et al., J. Virol., 62:1963-73 (1988) prepared two AAV vectors: dl 52-91, which retains the AAV rep genes, and dl 3-94 in which all of the AAV coding sequences are deleted. dl 3-94 does, however, retain the two 145 base terminal repeats and an additional 139 bases which contain the AAV polyadenylation signal. A foreign gene, encoding neomycin resistance, was inserted into the vector. Viral stocks were prepared by complementation with

a recombinant AAV genome which supplied the missing AAV gene products in trans but was itself too large to be packaged. Unfortunately, the virus stocks were contaminated with wild type AAV (10% in the case of dl 3-94) presumably as a result of homologous recombination between the defective and the complementing viruses.

Samulski et al., J. Virol., 63:3822-28 (1989) developed a method of producing recombinant AAV stocks without detectable wild-type helper AAV. The AAV vector retained only the terminal 191 bases of the AAV chromosome. In the AAV helper plasmid (pAAV/Ad), the terminal 191 bases of the AAV chromosome were replaced with adenovirus terminal sequences. Since sequence homology between the vector and the helper AAV thus essentially was eliminated, no detectable wild-type AAV was generated by homologous recombination. Moreover, the helper DNA itself was not replicated and encapsidated because the AAV termini are required for that process. Thus, in the AAV system, unlike the HSV system, helper virus could be eliminated completely leaving a helper-free AAV vector stock.

Recombinant AAV (rAAV) vectors have been used for expressing gene products in animals, see, for example, U.S. Pat. No. 5,193,941 and WO 94/13788. Other patents and publications describe AAV vectors and uses, the uses generally being related to expression of gene products either in vitro (usually tissue cultures) or in vivo (usually in the lungs or oral mucosa, the normal sites of AAV infection, but expression in other tissues, such as the central nervous system and in cardiac tissue has been observed).

Transduction of rAAV vectors harboring the bacterial β-galactosidase gene by single injection into the quadriceps of mice demonstrated that expression was maintained long-term and the expression did not decrease substantially during that time (Xiao et al., J. Virol., 70:8098-8108 (1996)). Other targets successfully transduced with rAAV vectors include: T-lymphocytes and B-lymphocytes, human

erythroleukemia cells, different regions of the rat brain, the striatum of the rat brain in a Parkinson's Disease model with the tyrosine hydroxylase gene, heart of the pig and rat with the LacZ gene, the peripheral auditory system of the guinea pig and bronchial epithelia of the rabbit and monkey. In addition, a Phase I human clinical trial for the delivery of an rAAV-CFTR construct is in progress.

AAV's harboring the human factor IX gene were infused into the portal vein of adult immunocompetent mice and long-term gene expression was obtained (Snyder et al., Nat. Genet., 16:270-276 (1997)). The vectors were found to be integrated into the murine genome (Miao et al., Nat. Genet., 19:13-15 (1998)).

II. HEMOPHILIA

Hemophilia A is an X chromosome-linked bleeding disorder resulting from a deficiency of or an abnormality of factor VIII (FVIII of fVIII), a component of the coagulation cascade. The human FVIII cDNA has been cloned. FVIII is synthesized as a 2351 amino acid residue, single chain precursor composed of a 19 amino acid signal peptide and six distinct domains. The domains are arranged in the order, A1-A2-B-A3-C1-C2 (Toole et al., Nature, 312:342-347 (1984); Vehar et al., Nature, 312:337-342 (1984)). An A domain contains about 330 amino acids and is present in three copies. A C domain contains about 150 amino acids and is present in two copies. The B domain contains about 909 amino acids and is extremely rich in potential N-linked glycosylation sites.

The translation product of the FVIII gene first is cleaved between the B domain and the A3 domain. Then, the B domain is proteolysed at multiple sites leaving FVIII as a divalent metal ion-linked complex consisting of the heavy chain (H chain) of 90-200 kDa and the light chain (L chain) of 80 kDa (Vehar et al., (1984), supra; Anderson et al., Proc. Natl. Acad. Sci. USA, 83:2979-2983 (1986)).

The minimal functional unit of FVIII is the heterodimer

consisting of the 90 kDa H chain and the 80 kDa L chain. Thus, the B domain is dispensable for procoagulant activity (Eaton et al., Biochemistry, 25:8343-8347 (1986); Toole et al., Proc. Natl. Acad. Sci., USA 83:5939-5942 (1986)). Circulating FVIII in blood is associated with the von Willebrand factor (vWF) which is a large multimeric, multifunctional product (Brinkhous et al., Proc. Natl. Acad. Sci. USA, 82:8752-8756 (1985)).

Hemophilia A patients are at risk of contracting transmissible infectious diseases from plasma-derived FVIII used in treatment. Thus, recombinant product is a desirable alternative. However, the complicated processing and large size of FVIII have hampered production of FVIII in prokaryotes or lower eukaryotes.

Expression of full-length FVIII cDNA in mammalian cells was reported by several groups, but the levels of expression were very low and insufficient for economical production of recombinant FVIII (rFVIII).

To improve expression efficiency, modified FVIII cDNA's lacking most of the B domain were made (Eaton et al., (1986), supra; Toole et al., (1986), supra; Sarver et al., Behring Inst. Mitt., 82:16-25 (1988); Meulien et al., Protein Engng., 2:301-306 (1988); Tajima et al., Proc. 6th Int. Symp., II.T:51-63 (1990)) and the resulting products were shown to retain functional activities of FVIII.

Tajima et al., (1990) supra, fused the coding sequences of the H and L chains. Although that construct was expressed about 10-fold higher than a full length FVIII cDNA construct, 20% of the product was not cleaved to the H and L chains or was cleaved incorrectly. Eaton et al. ((1986), supra) inserted a junction peptide derived from the B domain between the H and L chains. However, the junction peptide remained at the C terminus of the H chain. Such fusion molecules have antigenic properties (Esmon et al., Blood 76:1593-1600 (1990)) which can elicit serious side effects because of the constant exposure of the host to those antigens during the extended duration of treatment.

Burke et al. (J. Biol. Chem., 261:12574-12578 (1986)) expressed the H chain (Ala1-Arg740) and the L chain (Glu1649-Tyr2332) as separate proteins in COS cells and observed secretion of functionally active FVIII. But the expression levels were even lower than that of the full length construct. Yonemura et al. (Prot. Engng., 6:669-674, (1993)) used two plasmids to deliver H and L chain genes to CHO cells.

Another complication of the disease is the observation that the severity of the bleeding tendency varies among patients and may be related to the concentration of functional clotting factors. Individuals can have mild hemophilia that may not be recognized until adulthood or following heavy trauma or surgery, see, for example, Reiner & Davie, "Introduction to hemostasis and the vitamin K-dependent coagulation factors" in The Metabolic Basis of Inherited Disease (Scriver et al., eds.) Vol. 3, pages 3181-3221 (McGraw Hill, New York, 1995).

III. Therapy

Adenovirus vectors can infect non-dividing cells and therefore, can be delivered directly into mature tissue, such as muscle. However, the transgenes delivered by adenovirus vectors are not useful for long term expression for a variety of reasons. First, adenovirus vectors retain most of the viral genes and thus pose potential problems, i.e. safety. Expression of the adenovirus genes can cause the immune system to destroy the cells containing the vectors (see, for example, Yang et al., Proc. Natl. Acad. Sci., 91:4407-4411 (1994)). Since adenovirus is not an integration virus, the vector eventually will be diluted or degraded in the cells. Also, because of the immune response, adenovirus vectors cannot be delivered repeatedly. In the case of lifetime disease, such as the hemophilias, that will be a major limitation.

For retrovirus vectors, although stable integration into the host chromosomes can be achieved, the use thereof

is restricted because currently used vectors only can infect dividing cells, a large majority of target cells being non-dividing.

AAV vectors have certain advantages over the above-mentioned vector systems. First, like adenovirus, AAV infects non-dividing cells. Second, all the AAV viral genes are eliminated in the vector. Since the viral gene expression-induced immune reaction is no longer a concern, AAV vectors are safer than adenovirus vectors. As AAV is an integration virus, integration into the host chromosome will maintain the transgene in the cells. AAV is an extremely stable virus, resistant to many detergents, pH changes and heat (stable at 56°C for about an hour). AAV can be lyophilized and redissolved without losing significant activity. Finally, AAV causes no known diseases or pathogenic symptoms in humans. Therefore, AAV is a very promising delivery vehicle for gene therapy.

Two recent review articles provide an overview of the recent status on the use of AAV vectors and include a collection of additional recent scientific publications in the field: Samulski, "Adeno-associated Viral Vectors", Chap. 3 in "Viruses in Human Gene Therapy", Vos ed., Chapman & Hall, 1994; and Samulski, "Adeno-associated Virus-based Vectors for Human Gene Therapy", Chap. 11 in "Gene Therapy: From Laboratory to the Clinic", Hui ed., World Scientific, 1994.

Since AAV has in the past been shown to have a broad host range; can be administered by a variety of routes, including intramuscular injection; and is operable in different cells types, such as liver, retina, neurons and so on, there are no known limits of the host in which the herein described methods of delivery can take place, particularly in mammals, especially domesticated mammals such as cattle, sheep, pigs, horses, dogs, cats, chickens and turkeys.

SUMMARY OF THE INVENTION

The instant invention demonstrates for the first time that recombinant AAV vectors may be used to deliver for effective expression a protein with Factor VIII function to treat hemophilia A. Plural vectors deliver FVIII domains to host cells for expression and obtention of FVIII activity. The methods and recombinant AAV vectors described herein provide a significant development in the field of recombinant AAV vector gene therapy.

The methods and materials of the instant invention provide significant advantages, including the ability to deliver rAAV vectors harboring the genes to express at high levels a molecule with FVIII activity to, for example, hepatic or endothelial tissue via delivery routes such as, but not limited to, intraportal or intravenous administration in an intact animal.

The rAAV vectors of the invention can be used as nucleic acids or viral particles. The rAAV vector virus particles can be used alone or in conjunction with additional treatments, including partial hepatectomy or treatment with secondary agents that enhance transduction, whether associated with in vivo or ex vivo therapies. Examples of secondary agents include gamma irradiation, UV irradiation, tritiated nucleotides, such as thymidine, cis-platinum, etoposide, hydroxyurea, aphidicolin and adenovirus.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the rAAV-MFG-human-Factor VIII HC and rAAV-MFG-human-Factor VIII LC vectors. ITR: AAV inverted terminal repeat; MFG promoter: Murine Moloney Virus long terminal repeat; MLV IVS: mRNA splice donor/splice acceptor of murine leukemia virus; huF8HC: human Factor VIII heavy chain gene; huF8LC: human Factor VIII light chain gene; bGH pA: bovine growth hormone poly adenylation site.

FIG. 2 is a graph showing that functional factor VIII is made following infection of 293 cells in the presence of adenovirus with 1 x 10^{10} particles each of rAAV-MFG-human-Factor VIII HC and rAAV-MFG-human-Factor VIII LC vectors. Factor VIII activity was measured by COATest and ChromZ assays and is detected only when both vectors are present. GFP is an AAV vector expressing the green fluorescent protein described by Zolotukhin et al., J. Virol., 70:4646-4654 (1996), and served as a negative control.

FIG. 3 is a diagram of the pSSV9-MFG-S vector.

FIG. 4 is a diagram of the AAV-MFGSF8-LC vector.

FIG. 5 is a diagram of the AAV-MFGSF8-HC vector.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Because of the difficulty in cloning and expressing FVIII associated with the size of the FVIII precursor and the size of AAV vectors as well as the extensive processing of the precursor, it has been discovered that plural AAV vectors each carrying one or more FVIII domains can be used to obtain Factor VIII activity so long as at least the light (L) chain containing an A domain and two C domains and a heavy (H) chain containing two A domains are expressed. The plural AAV vectors can be infected into a single cell, despite the phenomenon of viral immunity, or different cells. A suitable approach is to use two vectors, one carrying a sequence encoding the FVIII heavy chain (HC) and the other carrying a sequence encoding the FVIII light chain (LC). However, it will be appreciated that other permutations are possible.

The rAAV vectors of the instant invention are derivatives of the adeno-associated virus, into which Factor VIII sequences have been introduced, of which various sequences have been modified.

While the wild-type adeno-associated virus is defective

in requiring helper virus for lytic infection, there is the possibility that the subject to whom the vector is delivered will harbor a herpesvirus or adenovirus infection which can complement the rAAV vectors and lead to production of rAAV particles. To guard against that possibility, the rAAV vectors are modified to reduce the possibility of rescue. In theory, such modifications can take the form of point mutations of one or more viral genes, which mutations either prevent expression of the gene altogether or result in the expression of a modified gene product which is nonfunctional. However, point mutations can be reversible. Consequently, it is preferable that each undesired adeno-associated virus gene simply be deleted, which has the additional advantage of creating more room within the viral package for larger nucleic acids containing FVIII sequences and/or regulatory elements.

It is preferable that all of the viral genes be deleted from the rAAV vectors, or otherwise inactivated, as in the known AAV vector dl3-94, see, e.g., McLaughlin, J. Virol., 62:1963-1973 (1988). However, it should be understood that an rAAV vector retaining one or more AAV genes, such as the known AAV vector, dl52-91, still may be useful for gene delivery, although possibly inferior to a preferred vector containing no functional AAV genes; see Hermonat, J. Virol., 51:329-339 (1984). Preferably, the rAAV vectors retain from AAV essentially only the recognition signals for replication and packaging (ITR).

For propagation of the rAAV vectors in vitro, susceptible cells are co-transfected with an AAV-derived vector DNA and a suitable AAV-derived helper virus or plasmid harboring the AAV rep gene, AAV cap gene or both and infected by a helper virus, including herpesvirus, adenovirus or a suitable helper plasmid. The particular method of producing viral particles is not critical to the invention. Any method of producing the rAAV viral particles can be used, including but not limited to that described in Samulski et al., (1989), supra, so long as appropriate

concentrations of viral particles capable of transducing cells in vivo and ex vivo are obtained. One of ordinary skill in the art will appreciate that any purification method used should produce infectious viral particles able to transduce, for example, hepatic cells or endothelial cells in vivo or ex vivo.

It is not necessary that the AAV-derived sequences correspond exactly with wild-type AAV prototypes. For example, the rAAV vectors of the instant invention may feature modified inverted terminal repeats and other sequences, provided that the rAAV vectors can replicate and be packaged with the assistance of helper virus, and establish a nonpathogenic latent infection in target cells.

The precise nature of regulatory regions needed for gene expression may vary from organism to organism, but in general, include a promoter which directs the initiation of RNA transcription in the cell of interest. Such regions may include those 5'-non-coding sequences involved with initiation of transcription, such as the TATA box. The promoter may be constitutive or regulated. Constitutive promoters are those which cause an operably linked gene to be expressed essentially at all times. Regulated promoters are those which can be activated or deactivated. Regulated promoters include inducible promoters, which are usually "off" but which may be induced to turn "on", and "repressible" promoters, which are usually "on" but may be turned "off". Many different regulators are known, including temperature, hormones, cytokines, heavy metals and regulatory proteins. The distinctions are not absolute; a constitutive promoter may be regulated to some degree.

The regulation of a promoter may be associated with a particular genetic element, often called an "operator", to which an inducer or repressor binds. The operator may be modified to alter the regulation thereof. Hybrid promoters may be constructed in which the operator of one promoter is transferred into another.

The promoter may be a "ubiquitous" promoter active in

essentially all cells of the host organism (e.g. the beta-actin or cytomegalovirus promoters) or may be a promoter with expression more or less specific to the target cells (such as the albumin promoter). Preferably, the tissue-specific promoters are essentially not active outside, for example, the hepatic system, and the activity of the promoter optionally may be higher in some components of the hepatic system than in others.

Thus, the promoter may be one which is active primarily in the hepatic system. The specificity may be absolute or relative. Similarly, the promoter may be specific for particular cell types, including but not limited to hepatocytes, Kupffer cells or endothelial cells.

In general, to locate a tissue-specific promoter, one identifies a gene which is expressed only (or primarily) in that tissue and then isolates the gene encoding that protein. (The gene may be a normal cellular gene or a viral gene of a virus which infects that cell.) The promoter of that gene is likely to retain the desired tissue-specific activity when linked to another gene. The tissue specificity of a promoter may be associated with a particular genetic element, which may be modified or transferred into a second promoter.

One of ordinary skill in the art will appreciate that a tissue-specific promoter for use in an AAV vector may be selected from any of the known liver-specific promoters and enhancers, including the albumin promoter, the alphafetoprotein promoter (Genbank Accession No. L34019), alphafetoprotein enhancer, human apolipoprotein E (ApoE) gene promoter and its associated liver-specific enhancers HCR-1 and HCR-2 (Nguyen et al., Oncogene, 12(10):2109-2119 (1996) and Allen et al., J. Biol. Chem., 270(44): 26278-26281 (1995)), the liver-specific enhancer of apolipoprotein AI (Malik et al., Mol. Cell. Biol., 16(4):1824-1831 (1996)) the factor VIII promoter, the factor IX promoter, the vWF promoter and the liver-specific human α1-antitrypsin promoter (Wu et al., Hum. Gene Therapy,

7(2):159-171 (1996) and Hafenrichter et al., Blood, 84(10):3394-3404 (1994)).

There also are other known strong promoters which find common use to obtain high levels of recombinant protein expression. For example, the herpes simplex thymidine kinase promoter, SV40 promoter and LTR's have found wide use as strong promoters. An example is the LTR obtained from Moloney leukemia retrovirus.

For the gene to be expressed, the coding sequence must be operably linked to a promoter sequence functional in the target cell. A promoter region would be linked operably to a coding sequence if the promoter were positioned so that, when the promoter was activated, the coding sequence was transcribed. The coding sequences are linked operably if the linkage does not cause an error in the reading of the downstream sequence. To be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

The rAAV vectors may further comprise one or more restriction sites into which polynucleotides carrying an FVIII domain or domains may be cloned without interfering with packaging and replication. Preferably, at least one unique restriction site is provided. The rAAV vectors also may comprise one or more marker genes to facilitate genetic manipulation. Suitable marker genes are known in the art and include, but are not limited to, the neomycin and hygromycin resistance genes, bacterial lacZ and the firefly luciferase gene.

As to optional mRNA slice donor/splice acceptor sequences associated with intervening sequences, any such donor/acceptor sequence compatible with and operable in AAV can be used. Particular, non-limiting examples of suitable donor/acceptable sites are taught herein and are known in the art.

As to polyadenylation sites, any such polyadenylation site compatible with and operable in AAV can be used. Particular, non-limiting examples of suitable

polyadenylation sites are taught herein and all known in the art.

If desired, the non-coding region 3' to the gene sequence coding for the desired RNA product may be obtained. The region may be retained for its transcriptional termination regulatory sequences, such as those which provide for termination and polyadenylation. Thus, by retaining the 3' region naturally contiguous to the coding sequence of the FVIII light chain, the transcriptional termination signals may be provided. Where the transcriptional termination signals natively associated with the coding sequence are not satisfactorily functional in the expression host cell, then a different 3' region, functional in the host cell, may be substituted.

As to the AAV inverted terminal repeats, any such ITR's or derivatives thereof, which contain nucleotide substitutions, deletions, inversions and/or insertions yet retain the requisite biological activities required of an AAV vector carrying a foreign gene, can be used. Also, ITR's from different AAV serotypes can be used.

Another element which contributes to the proper of expression of FVIII is a signal sequence. A suitable signal sequence is that of native FVIII. Thus, the native FVIII signal sequence can be cloned upstream of the N-terminal amino acid of the FVIII domain sequences in the plural vector constructs.

Typically, because of the packaging limitations of AAV, the polynucleotides encoding FVIII domain sequences and regulatory sequences can have a length of up to about 5,500 bases.

The AAV helper virus or AAV helper plasmid may be any virus or plasmid which is capable, on expression of the AAV genes it carries, of providing proteins necessary for the replication and packaging of the rAAV vector in a suitable host cell, for the purpose of producing rAAV vector stock.

The non-AAV helper virus or non-AAV helper plasmid is one which has been engineered to reduce the risk of

recombination between the non-AAV helper DNA and the rAAV vector DNA.

Thus, it is preferable there is very limited or no sequence homology between the AAV sequences of the vector DNA and the AAV sequences of the helper DNA. For example, the helper DNA may be an AAV sequence in which the AAV inverted terminal repeats are replaced by the corresponding sequences of another virus, such as adenovirus (e.g., adenovirus type 5 DNA), see Samulski et al., (1989), supra.

Also, the non-AAV helper virus may be one in which a gene necessary for replication is made defective. For example, an adenovirus stock wherein a gene thereof, such as the E1A gene, is disabled is desirable.

Non-AAV helper virus may be removed by heat inactivation at about 56°C for about 30-45 minutes, or physically separated from packaged rAAV vectors by any of a variety of methods, including single or multiple centrifugation runs in cesium chloride.

Basic procedures for constructing recombinant DNA and RNA molecules in accordance with the instant invention are disclosed in numerous publications, including Sambrook et al., In: Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), which is herein incorporated by reference.

The instant invention may be used for gene therapy of Factor VIII associated disorders, such as hemophilia A. An individual may be in need of gene therapy because, as a result of one or more mutations in the regulatory region and/or the coding sequence of the Factor VIII gene, Factor VIII is expressed inappropriately, e.g., has an incorrect amino acid sequence, is expressed in the wrong tissues or at the wrong times, is underexpressed or overexpressed.

The target cells of the vectors of the instant invention are cells capable of expressing polypeptides with FVIII activity, such as those of the hepatic system of a mammal, endothelial cells and other cells with the proper

machinery to process the precursor to yield protein with factor VIII activity and to secrete same. In one embodiment, the cells are normal cells cultured in vitro. The target cells may be human cells or cells of other mammals, such as, nonhuman primates, rodents, carnivores and other domesticated species.

In another embodiment, the cells are part of a living mammal at the time the rAAV vectors are delivered to the cell. The mammal may be at any stage of development at the time of delivery, e.g., embryonic, fetal, infantile, juvenile or adult. Additionally, the cells may be healthy or diseased.

To deliver the vectors specifically to a particular region of, for example, the liver, they may be administered by intraportal injection. Because the AAV vectors will be maintained stably in the target cells, rather than producing viral particles, the subsequent spread of the vector will be minor and will be mainly a function of passive diffusion from the site of injection. The degree of diffusion may be controlled by adjusting the ratio of rAAV vectors to fluid carrier.

In certain embodiments, the vectors will be administered via an intravascular approach. For example, the vectors can be administered intra-arterially. Of course, with intravenous as well as intraportal delivery, the recipient mammal must be able to tolerate the possibility of delivery of the vectors to cells other than those of interest, such as, of the hepatic system.

For targeting the vectors to a particular type of cell, e.g., hepatocytes, it may be beneficial to associate the vector with a homing agent that binds specifically to a surface receptor of the cell. Thus, the vectors may be conjugated to a ligand (e.g., galactose) for which certain hepatic system cells have receptors. The conjugation may be covalent, e.g., using a crosslinking agent (e.g. glutaraldehyde) or noncovalent, e.g., the binding of an avidinated ligand to a biotinylated vector. Another form of

covalent conjugation is provided by engineering the AAV helper plasmid used to prepare the vector stock so that one or more of the encoded coat proteins is a hybrid of a native AAV coat protein and a peptide or protein ligand, such that the ligand is exposed on the surface of the viral particle.

Whatever the form of conjugation, it must not interfere substantially either with the production or transduction of the rAAV vectors.

The use of plural rAAV's to transduce single cells may depend on whether the host cell is multinucleate, polysomic or polyploid. Thus, other suitable target cells may be cells of a syncytium, muscle cells, germ cells, marrow cells, megakaryocytes and so on. However, the instant invention is not to be construed to be limited to such cells and may be practiced on normal, diploid, somatic, mononucleate cells as well, so long as proper processing and expression of polypeptides with Factor VIII activity occurs.

As used herein, a multinucleate cell is one which contains more than one nucleus. A multinucleate cell may be polyploid or polysomic, in whole or in part. The development of more than one nucleus in a cell is not necessarily dependent on having multiple, complete diploid sets of chromosomes. Often, however, a multinucleate cell contains multiple, complete diploid sets of chromosomes.

The rAAV vectors may be administered as viral particles alone, whether as an in vivo direct delivery to the portal vasculature or as an ex vivo treatment comprising administering the rAAV vector viral particles in vitro to cells from the animal receiving treatment followed by introduction of the transduced cells back into the donor. Alternatively, the rAAV vector virus particles can be used to transduce cells in conjunction with secondary agents known to enhance the efficiency of transduction, see, e.g., WO 95/33824, using any of a variety of secondary agents. Secondary agents useful for enhancing transduction efficiency include radioactive molecules, including tritiated nucleotides, ultraviolet radiation, gamma

irradiation, cis-platinum, hydroxyurea, etoposide, camptothecin, aphidicolin and adenovirus, see e.g., Ferrari et al., J. Virol., 70:3227-3234 (1996).

Prior to administration to a host, it is beneficial to determine the purity of the recombinant AAV preparation, that is, the AAV vector containing the transgene. For example, while no diseases are associated with AAV infection, knowing the degree of wild-type AAV contamination in a recombinant virus preparation is desirable. Wild-type virus can be generated by crossover between, for example, the AAV helper plasmid and the AAV plasmid carrying the transgene.

The presence of contaminating wild-type AAV can be determined, for example, by a nucleic acid amplification assay, such as PCR or an RNA based amplification method such as 3SR (Gingeras et al., Ann. Biol. Clin., 48:498-501 (1990)) and NASBA (van der Vleit et al., J. Gen. Micro., 139:2423-2429 (1993)).

Thus, in the case of PCR, AAV nucleic acid is prepared and subjected to a PCR reaction, along with positive and negative controls. The strategic identification of certain PCR primers enables distinguishing wild-type from recombinant virus in an expeditious and efficient fashion. Thus, primers are selected to be specific for wild-type AAV or for wild-type AAV derived through recombination of the helper and vector plasmids.

AAV vectors derived from pSub201 (Samulski et al., J. Virol., 61:3096-3101 (1987)) or other vectors tailored to have distinctive sequences in the left and right arms of the vector enable distinguishing wild-type virus from wild-type virus arising from recombination. By left and right arms, it is meant to refer to those portions of the vector which flank the cloning site which contains the transgene. Thus, in the case of pSub201, the left arm is that portion to the left of the XbaI site and the right arm is that portion to the right of the XbaI site. The arms contain the hairpin structures.

Referring to the exemplified pSub201 vector to explain the method, it is noted that pSub201 has sequences found normally on the right arm of AAV on both sides of the transgene. Thus, sequences found normally only on the right arm are present on the left arm as well, the native left sequences having been deleted.

Accordingly, primers can be configured wherein the presence or absence of left sequences or the presence of right sequences on both sides of the virus can be used to distinguish wild-type AAV containing the normally occurring left sequences from any wild-type AAV generated by recombination between the helper and vector plasmids containing right sequences on both sides of the transgene.

For example, when using the pSub201 vector, or equivalent vector containing right arm sequences on both arms, the primers which hybridize to sequences in the AAV ITR in the AAV rep gene; in the AAV splice region; or in the AAV cap gene can be diagnostic.

As to the primers, it will be well appreciated that once suitable sites are located in the two genomes which are found to be diagnostic of wild-type and crossover-generated wild-type virus, the exact nucleotide sequence and length of any one primer can be varied without detracting from the object of the diagnostic assay. The limitations to the variations to the primers depend on, for example, the reaction conditions of PCR to assure hybridization of the primer to target.

In the case of NASBA or 3SR, essentially the same primers can be used and which will be configured to contain a suitable RNA polymerase promoter. Another primer for synthesis of the double-stranded intermediate can rely on, for example, use of the Cap2 or AAV2S2 primers.

The instant invention includes pharmacological intervention in vivo or ex vivo to treat FVIII-related disorders. The rAAV vectors, which can comprise the rAAV vectors (including the ITR's) packaged in viral particles, are administered to a subject in an amount effective to

obtain the desired Factor VIII activity in serum.

Administration can be by any means in which the therapeutic polynucleotides are delivered to the desired target cells. For example, both in vivo and ex vivo methods are contemplated. Intravenous injection of rAAV vectors to the portal vein is a suitable method of administration for transducing liver cells. Other in vivo methods include, for example, direct injection into the lobes of the liver or the biliary duct and intravenous injection distal to the liver. Ex vivo modes of administration include transduction in vitro of resected hepatocytes or other cells of the liver with the rAAV vectors, followed by infusion of the transduced, resected hepatocytes back into the portal vasculature or biliary tree of the human patient, see e.g., Grossman et al., Nature Genetics, 6:335-341 (1994).

Whether the transduction of liver cells occurs in vivo or ex vivo, the rAAV vector virus particles can be delivered either alone or in conjunction with a partial hepatectomy, a helper virus (including, for example, adenovirus, CMV or HSV-1) or a secondary agent for enhancing transduction efficiency.

The effective amount of rAAV vectors to be administered will vary from patient to patient. Accordingly, effective amounts are best determined by the physician administering the rAAV vectors and appropriate dosages can be determined readily by one of ordinary skill in the art. A useful initial amount for administration may be in the range from 10^9 to 10^{20} particles and perhaps 10^9 to 10^{15} particles of each vector for a 70 kg adult. Generally, the minimum effective amount of virus is desirable. After allowing sufficient time for the rAAV vectors to be expressed (typically 4-15 days, for example), analysis of the serum or other tissue for Factor VIII activity and comparison to the initial level prior to administration will determine whether the amount being administered is too low, within the right range or too high.

Suitable regimes for initial and subsequent

administrations also may be variable, but are typified by an initial administration followed by subsequent administrations, if necessary. Subsequent administrations may be administered at variable intervals, ranging from daily to annually to every several years. One of skill in the art will appreciate that appropriate immunosuppressive techniques may be recommended to avoid inhibition or blockage of transduction by immunosuppression of the rAAV viral vectors, see e.g., Vilquin et al., Human Gene Ther., 6:1391-1401 (1995).

Formulations for both ex vivo and in vivo administrations include suspensions in liquid or emulsified liquids. The active ingredient (rAAV vector) often is mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol or the like, and combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances, such as, wetting or emulsifying agents, pH buffering agents, stabilizing agents or other reagents that enhance the effectiveness of the pharmaceutical composition.

Pharmaceutical compositions can be prepared as injectable formulations for administration as known in the art, including the use of implantable pumps (known by those of skill in the art and described, for example, in U.S. Pat. No. 5,474,552). Numerous formulations for oral or parenteral administration are known and can be used in the practice of the instant invention. The vectors can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

For parenteral administration, solutions in an adjuvant such as sesame or peanut oil or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of the AAV vector as a free acid (DNA contains acidic phosphate groups) or a pharmacologically

acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion of AAV viral particles also can be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, the preparations contain a preservative to prevent the growth of microorganisms. The sterile aqueous media employed are obtainable by standard techniques well known to those skilled in the art.

The pharmaceutical forms suitable for parenteral administration include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that parenteral administration is possible. The formulation must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile parenteral formulations are prepared by incorporating the AAV vector in the required amount in the appropriate solvent with various of the other ingredients

enumerated above, as required, followed by sterilization, such as by filtration. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze drying which yield a powder of the active ingredient plus any additional desired ingredient.

The instant recombinant vectors also can be administered orally. Such preparations can be formulated as known in the art and can include, for example, flavorants, odorants, colorings and the like to facilitate presentation.

The invention now being generally described, the same will be better understood by reference to the following examples, which are provided for purposes of illustration only and are not to be considered limiting of the invention.

EXAMPLES

AAVMFGSF8-HC is an AAV vector in which the human FVIII heavy chain coding sequences (followed by introduced translational step codons) are under the transcriptional control of the MLV LTR and intron from MFG (Dranoff et al., PNAS, 90:3539-3543 (1993)). Polyadenylation sequences are derived from the bovine growth hormone (bGH) gene.

AAVMFGSF8-LC is an AAV vector in which the coding sequences for the human FVIII light chain replace the human FVIII heavy chain sequences of AAVMFGSF8-HC. Both vectors contain the signal sequence of human FVIII heavy chain.

AAVMFGSF8-HC was generated by ligating BamHI and NcoI digested pSSV9-MFG-S with a 1.8 kb NcoI-Asp718 fragment from MFG-hFVIIIΔB (Dwarki et al., PNAS, 92:1023-1027 (1995)) and a .48 kb Asp718-BamHI fragment from pTAF8-HC. pTAF8-HC was derived by ligating TA cloning vector pCR2.1 (Invitrogen) with a fragment generated by PCR using MFG-hFVIIIΔB as the template with oligonucleotide primers

5'-AAGCTGGTACCTCACAGAA-3' (SEQ ID NO:1) and 5'-TCGATGGATCCTCATTAATTCTGGGAGAAGCTTCTTGG-3' (SEQ ID NO:2).

AAVMFGSF8-LC was generated by ligating BamHI and AgeI digested pSSV9-MFG-S with a .26 kb AgeI-ScaI fragment from pTasig, a .78 kb HincII-NdeI fragment from pTAF8-LC and a 1.54 kb NdeI-BamHI fragment from MFG-hFVIIIΔB. pTasig, containing the FVIII signal sequence followed by a ScaI site was derived by ligating the TA cloning vector pCR2.1 (Invitrogen) with a fragment generated by PCR using MFG-hFVIIIΔB as the template with oligonucleotide primers 5'-ACAGGCTCTCTAACTTAGT-3'(SEQ ID NO:3) and 5'-TTAGCAGTACTAAAGCAGAATCGCAAAA-3' (SEQ ID NO:4). pTAF8-LC contains the FVIII light chain coding sequences, preceded by a HincII site, was derived by ligating the TA cloning vector pCR2.1 (Invitrogen) with a fragment generated by PCR using MFG-hFVIIIΔB as the template with oligonucleotide primers 5'-GTCGACCCTCTTGCTTGG-3' (SEQ ID NO:5) and 5'-AATTCCCATATGATGTTGCACTTT-3'(SEQ ID NO:6).

The recombinant AAV vectors were packaged as described by Snyder et al., in Current Protocols in Human Genetics, pp. 12.1.1-12.1.24, Dracopoli et al., eds., John Wiley & Sons, New York, 1996.

To create rAAV viral vectors, the plasmids were co-transfected along with an AAV helper plasmid (which provides necessary AAV replication and structural proteins but lacks AAV termini and thus cannot package into virus) via the calcium phosphate method into 293 cells (Graham et al., J. Gen. Virol., 36:59-74 (1977)), which constitutively express the adenovirus E1a protein. A suitable helper plasmid is pDCG2-1 (Li et al., J. Virol., 71:5236-5343 (1997)). The next day, the co-transfected 293 cells were infected with an adenovirus type 5 strain, such as, adenovirus strain dl312 or dl309 (Jones & Shenk, Cell, 17:683-689 (1979)) to provide remaining replication and packaging machinery. Following full cytopathic effect, virus was harvested by multiple freeze/thaw cycles and purified according to Snyder et al., (1996), supra. A

suitable nuclease to remove unwanted nucleic acids is Benzonase (Nycomed, Copenhagen, Denmark) at 250U/ml. The resulting viral stock consisted of packaged rAAV vectors and progeny helper adenovirus. The helper virus was eliminated by purification on cesium chloride (CsCl) density gradients. Viral stocks then were heated to 56°C for 30 min. to inactivate residual helper adenovirus (Samulski et al., (1989), supra). Vector titers are obtained by dot-blot hybridization (Snyder et al., (1996), supra).

The culture medium was removed and analyzed for human Factor VIII activity by known methods, such as, an ELISA (Connelly et al., Blood, 87:4671-4677 (1996)) and functional tests, such as, COATest (KabiVitrum, Stockholm, Sweden).

Immunoreactive factor VIII and factor VIII activity were observed.

All references mentioned in the instant specification are herein incorporated by reference in entirety.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of obtaining human factor VIII activity in a subject, comprising:

administering a plurality of recombinant adeno-associated virus (rAAV) vectors to cells capable of expressing factor VIII,

wherein each of said vectors express one or more domains of human factor VIII and said expressed domains comprise a light chain and a heavy chain of factor VIII without a B domain.

2. The method of claim 1, wherein said subject is a mammal.

3. The method of claim 1, wherein liver cells of said subject are transduced with said rAAV vectors ex vivo, and said administering further comprises delivering said transduced liver cells into the portal vasculature of said mammal.

4. The method of claim 3, wherein said subject is a mammal.

5. The method of claim 1, wherein a first vector expresses human factor VIII heavy chain and a second vector expresses human factor VIII light chain.

6. The method of claim 1, wherein said rAAV vectors comprise:

a promoter capable of expressing human factor VIII,

a structural gene encoding one or more domains of human factor VIII and

two AAV inverted terminal repeats,

wherein said inverted terminal repeats flank the promoter and structural gene.

7. The method of claim 6, wherein said inverted terminal repeats comprise a portion of wild-type AAV inverted terminal repeats.

8. The method of claim 6, wherein said promoter is obtained from a virus.

9. The method of claim 8, wherein said virus is murine leukemia virus.

10. The method of claim 9, wherein said murine virus is Moloney murine leukemia virus.

11. The method of claim 6, wherein said rAAV vectors further comprise a signal peptide sequence upstream of said structural gene.

12. The method of claim 11, wherein said signal peptide sequence is the human factor VIII signal peptide sequence.

13. The method of claim 1, further comprising:
providing said subject with a partial hepatectomy.

14. The method of claim 1, further comprising:
administering a helper virus into the portal vasculature of said subject.

15. The method of claim 1, further comprising:
administering a secondary agent for enhancing transduction efficiency to said liver cells of said subject.

16. The method of claim 15, wherein said administering of said secondary agent and said administering of said recombinant AAV vector occur in vivo.

17. The method of claim 3, wherein a secondary agent is applied to said liver cells of said subject to enhance

transduction with said recombinant AAV vector ex vivo.

18. The method of claim 1, wherein said administering comprises:

injecting said recombinant AAV vectors into the portal vasculature of said subject.

19. The method of claim 18, wherein said subject is a mammal.

20. The method of claim 1, wherein factor VIII activity is detectable at an elevated level in blood of the subject as compared to factor VIII activity in blood of said subject prior to said administering step.

21. The method of claim 3, wherein said liver cell is a hepatocyte.

22. The method of claim 6, wherein a first rAAV vector expresses human factor VIII heavy chain and a second rAAV vector expresses human factor VIII light chain.

23. A pharmaceutical composition comprising:

a plurality of recombinant adeno-associated (rAAV) vectors each comprising a polynucleotide encoding one or more domains of human factor VIII operably linked to a promoter and a polyadenylation sequence; and

a pharmaceutically acceptable, carrier, excipient or diluent,

wherein said plurality of rAAV vector express a heavy chain and a light chain of factor VIII without a B domain.

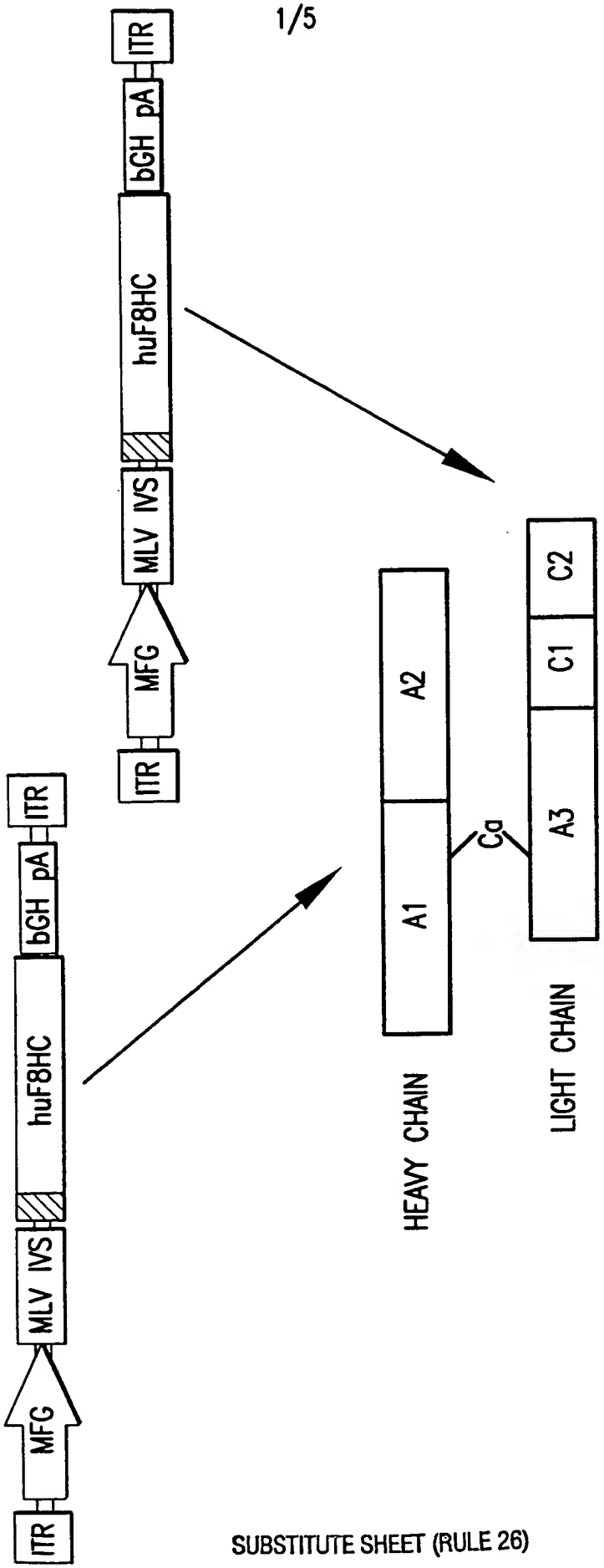
24. The composition of claim 23, wherein said promoter is obtained from a virus.

25. The composition of claim 24, wherein said virus is murine leukemia virus.

26. The composition of claim 25, wherein said virus is Moloney murine leukemia virus.

27. The composition of claim 23, wherein said recombinant AAV vectors further comprise a signal peptide sequence upstream from said polynucleotide encoding one or more domains of human factor VIII.

28. The composition of claim 27 wherein said signal sequence is the human factor VIII signal peptide sequence.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Co
C2
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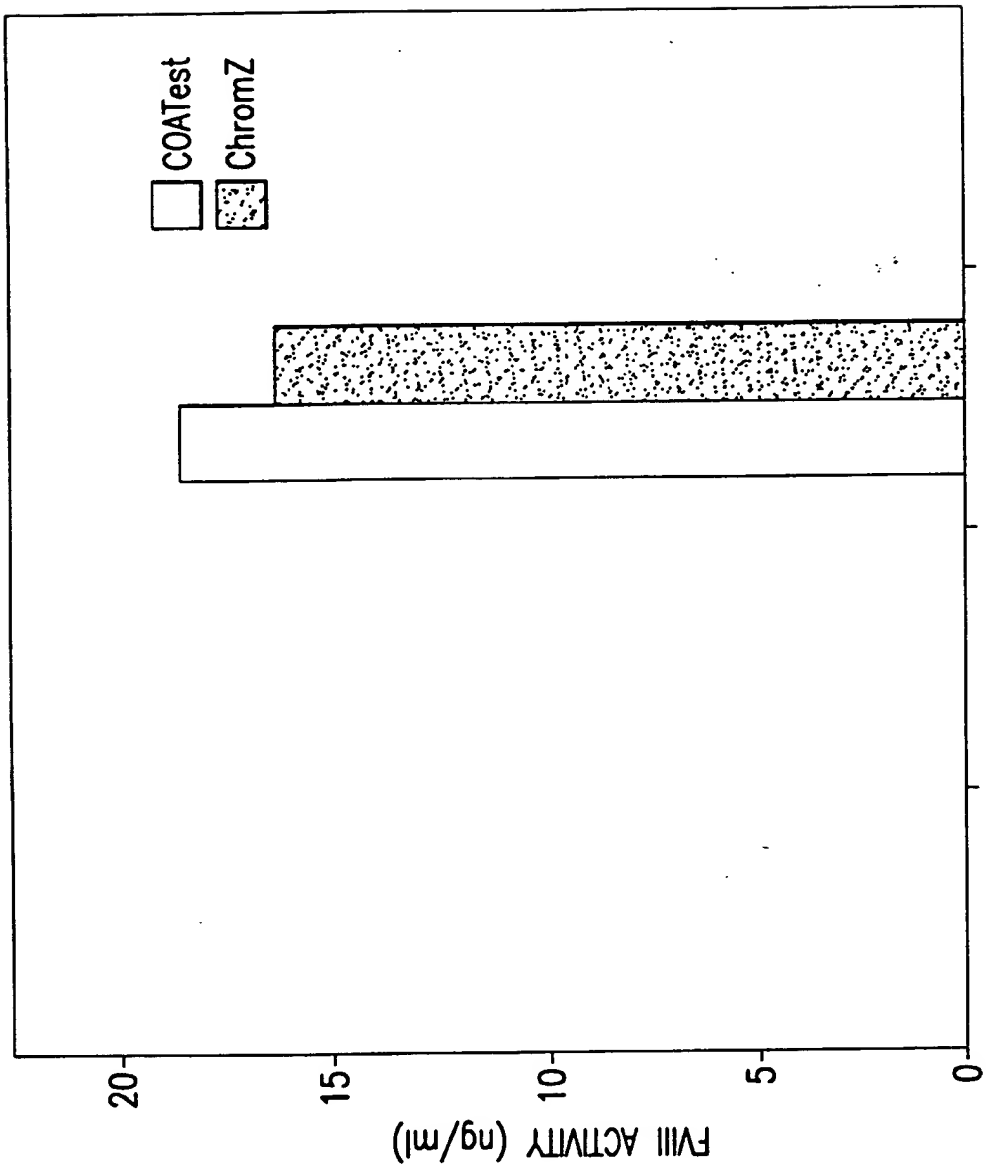
HEAVY CHAIN
LIGHT CHAIN

FIG.1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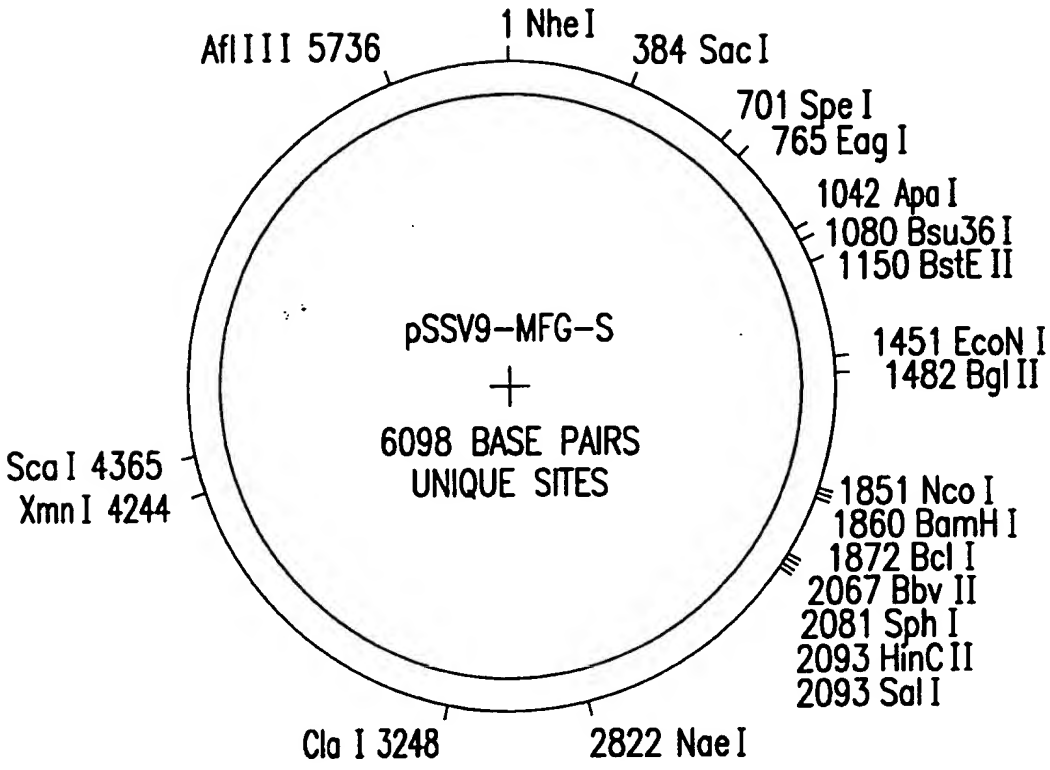
VECTOR
LC + HC
GFP

FIG.2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1042 Apa I
1080 Bsu36 I
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1482 Bgl II
1851 Nco I
1860 BamH I
1872 Bcl I
2067 Bbv II
2081 Sph I
2093 HinC II
2093 Sal I
2822 Nae I
Cla I 3248
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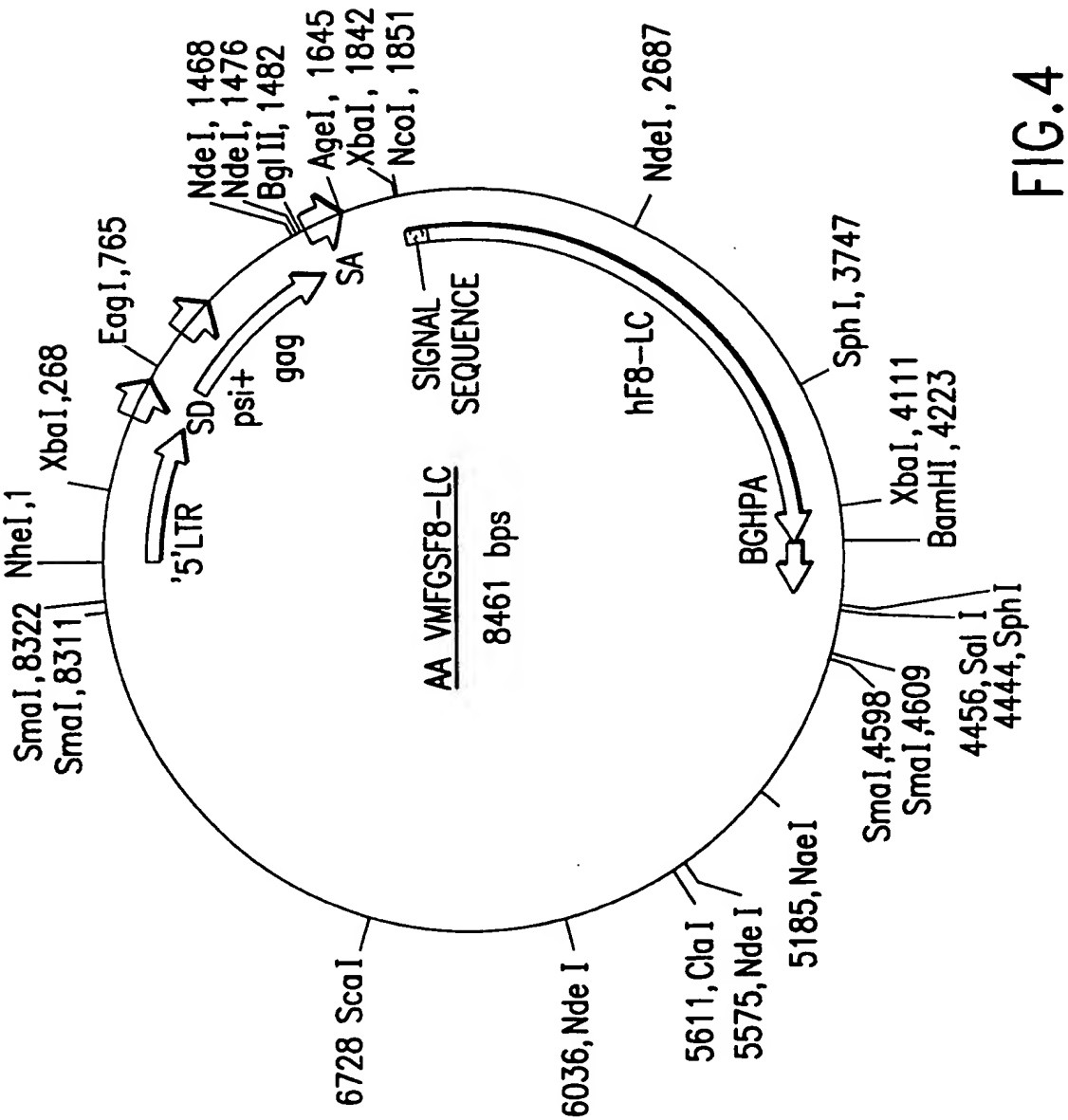
Afl III 5736
pSSV9-MFG-S
6098 BASE PAIRS
UNIQUE SITES

FIG.3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4456, SalI
4444, SphI
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5185, NaeI
5611, ClaI
5575, NdeI
6036, NdeI
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SmaI, 8322
SmaI, 8311
NheI, 1
XbaI, 268
EagI, 765
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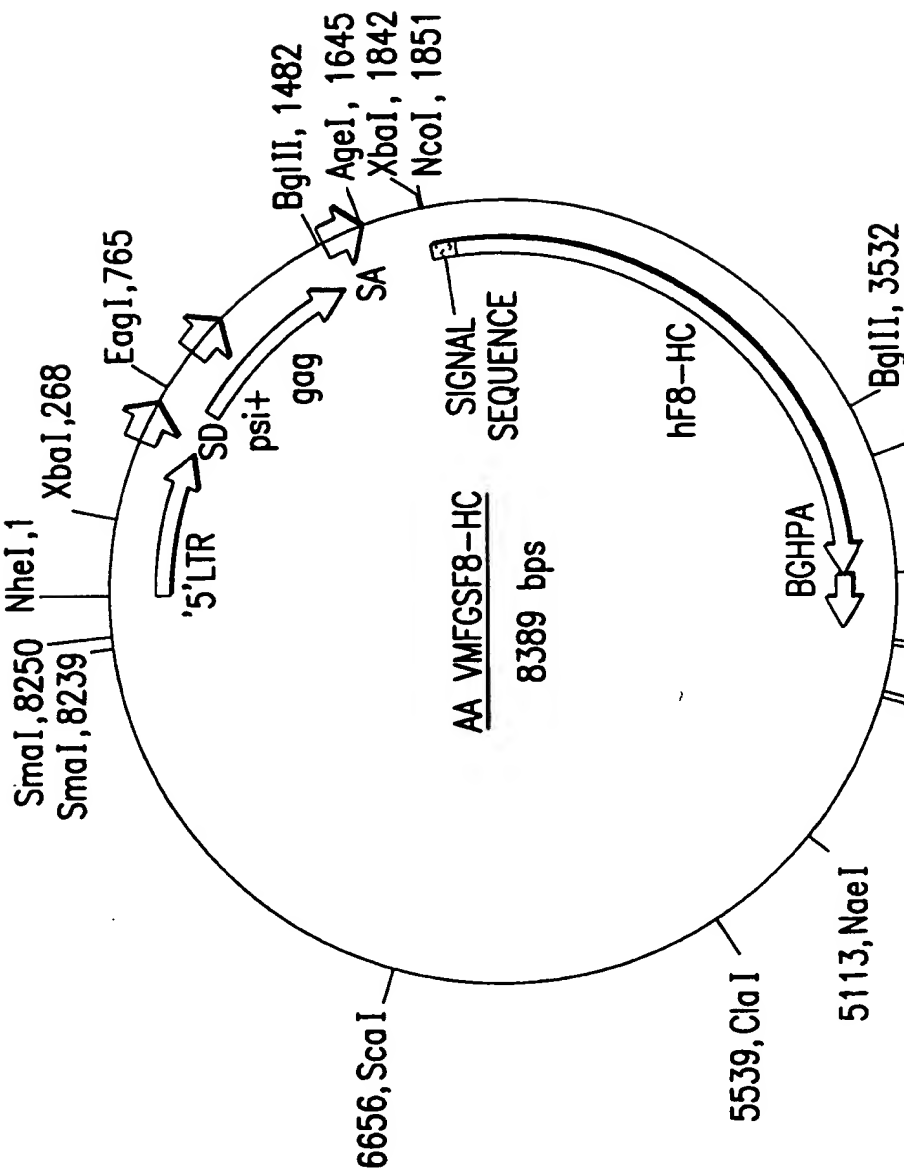
FIG. 4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SA
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BamHI, 3721
BamHI, 4151
4384, Sal I
4372, SphI
Sma1,4526
Sma1,4537
5113, NaeI
5539, Cla I
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gag
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BGHPA

FIG.5

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<141>

<150> 09/084,423
<151> 1998-05-27

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International Bureau

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(54) Title: **ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED EXPRESSION OF FACTOR VIII ACTIVITY**
(54) Titre: **EXPRESSION DE L'ACTIVITE DU FACTEUR VIII PAR UN VECTEUR VIRAL ADENO-ASSOCIE**

(57) Abstract

The instant invention provides methods and materials for expressing polypeptides with factor VIII activity comprising administering at least two rAAV vectors encoding different domains of human factor VIII and at least the heavy and light chains.

(57) Abrégé

L'invention concerne des procédés et des matériaux permettant l'expression de polypeptides comprenant une activité facteur VIII. Ces procédés comprennent l'administration d'au moins deux vecteur rAAV codant pour différents domaines du facteur VIII humain et au moins pour les chaînes lourde et légère.

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(71) Applicant: CELL GENESYS, INC. [US/US]; 342 Lakeside Drive, Foster City, CA 84404 (US).

(72) Inventors: COHEN, Lawrence, K.; 10065 Broadway Terrace, Oakland, CA 94611 (US). SPRATT, S., Kaye; 104 Edmondale Court, Vacarille, CA 95688 (US). COUTO, Linda; 7814 Oak Creek Drive, Pleasonton, CA 94588 (US).

(74) Agents: NAKAMURA, Dean, H. et al.; Sughrue, Mion, Zinn, MacPeak & Seas, PLLC, Suite 800, 2100 Pennsylvania Avenue, N.W., Washington, DC 20037-3202 (US).

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(57) Abstract

The instant invention provides methods and materials for expressing polypeptides with factor VIII activity comprising administering at least two rAAV vectors encoding different domains of human factor VIII and at least the heavy and light chains.

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INTERNATIONAL SEARCH REPORT

Int tional Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 A61K48/00

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GNATENKO, D. ET AL.: "Recombinant adeno-associated virus as a vehicle for gene delivery of human mutant factor VIII" BLOOD, vol. 90, no. 10 Suppl., 1 Part 1, 15 November 1997 (1997-11-15), page p119A XP002122884 see the abstract — -/—	1,6,7

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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